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The Enzymatic Microestimation of Urea

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An enzymatic micromethod for estimating urea in serum and urine is described. Urea is first converted by urease into ammonia which is then estimated by the reaction with α-ketoglutarate catalysed by glutamic dehydrogenase. No deproteinization or dialysis of the serum is required.

Es wird eine enzymatische Mikromethode für die Bestimmung von Harnstoff in Serum und Urin beschrieben. Nach Hydrolyse mit Urease (Urea Amidohydrolase EC 3.5.1.5) wird das Ammoniak mit Glutamatdehydrogenase (L-Glutamat: NAD Oxydoreduktase, desaminierend, EC 1.4.1.2) bestimmt. Enteiweißung oder Dialyse des Serums ist nicht erforderlich.

Fawaz and v. Dahl (1) and Kirsten, Gerez and Kirsten (2) published an enzymatic method for the estimation of ammonia in tissues and body fluids. Mondzac, Ehrlich and Seegmiller (3), apparently unaware of the above publications, also described the same procedure. The method is based on the reaction:

$$NH_4^+ + H^+ + NADH + \alpha\text{-ketoglutarate} \xrightarrow{\text{glutamic dehydrogenase}} \text{L-glutamate} + NAD^+ + H_2O.$$

Since the reaction goes to completion under experimental conditions, the decrease in extinction (ΔE) at 340 nm is a measure of the amount of NAD formed and thus of the quantity of ammonia present. This enzymatic method is not only sensitive, but also specific, since related amines like methyl- and ethylamines do not react. Furthermore, the method does not involve the use of strong alkali which could result in the de novo synthesis of extra ammonia.

For the estimation of blood ammonia, protein-free filtrates from heparinized plasma are used. The use of serum without deproteinization, presents difficulties which have not yet been surmounted. However, since the concentration of urea nitrogen in blood exceeds that of ammonia nitrogen several hundred-fold, it was thought possible to use *native* serum in minute quantities for the estimation of urea. Ammonia, liberated from urea by the action of urease, could then be determined by the glutamic dehydrogenase catalysed reaction. Such a method is described in this paper. It permits the estimation of urea in as little as one microliter of serum and avoids the disadvantages inherent in color reactions such as nesslerization etc.

Reagents

All reagents are dissolved in glass-redistilled water made ammonia-free by redistillation following acidification with sulfuric acid.

1. Glutamic dehydrogenase (Boehringer) dissolved in glycerol, 10 mg/m*l*.

2. KH_2PO_4 — Na_2HPO_4 buffer, pH 7.6, 0.2M.

3. NADH Na_2 (Boehringer) 0.01M, dissolved in neutral ammonia-free water freshly prepared.

4. Adenosine diphosphate (ADP $Na_3 \cdot H_2O$ Boehringer), 10 mg/m*l*. (The purpose of the ADP is to protect glutamic dehydrogenase from inactivation due to depolymerization.)

5. α-ketoglutaric acid (0.2M), carefully neutralized with 0.4N NaOH.

6. Urease (Boehringer, 5.34 U/mg). Shake 100 mg of urease powder with 5 m*l* 50% aqueous glycerol and centrifuge off undissolved particles. The urease solution contains significant amounts of ammonia which can be removed by treatment with permutit. Shake with 500 mg permutit for 10 min. and centrifuge. Repeat the permutit treatment twice. The solution is stored in the refrigerator at 3—5° C.

Procedure

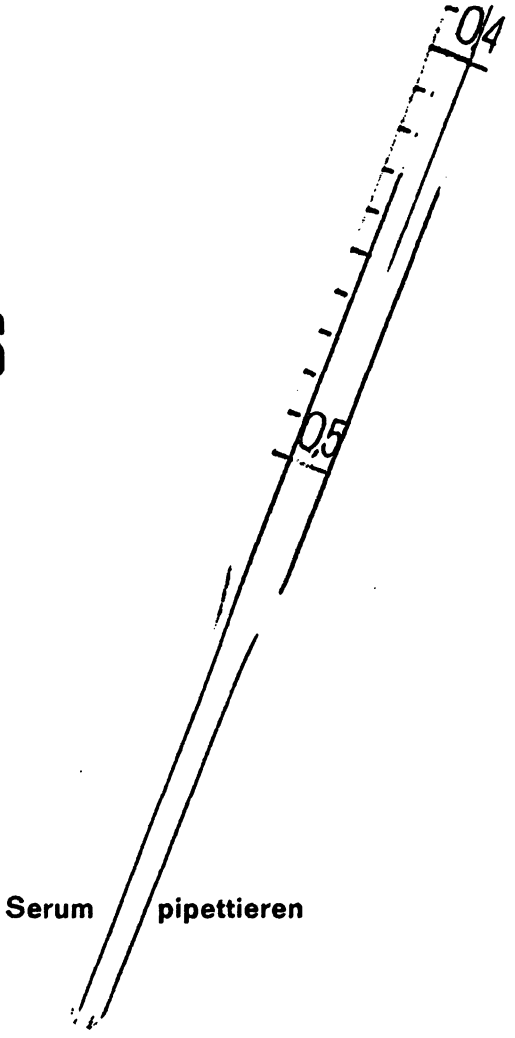
Native serum (without deproteinization) is used. It is diluted 1:10 with the phosphate buffer when the urea content is known to be within normal limits. In patients known to be uremic greater dilution is required and the volume of the sample is chosen to give a decrease in optical density (ΔE) of 0.2—0.6. The method is also used to measure urea (and ammonia) in urine. For this purpose, urine is diluted 1:100 and a sample of 0.04—0.08 m*l* is used for ammonia estimation. It is diluted 1:500 for urea estimation and the volume of the sample is 0.03—0.06 m*l*.

Reagents are pipetted into a semi-micro cuvette (light path 1 cm, width 0.4 cm) of a Beckman B spectrophotometer or an equivalent instrument, in the following order:

0.5 m*l* phosphate buffer (Final concentrations 100 mM).
0.01 m*l* NADH solution (Final concentration 0.1 mM).
0.025 m*l* ketoglutarate solution (Final concentration 5.0 mM).
0.01 m*l* ADP solution (Final concentration 0.2 mM).
0.04 m*l* diluted (normal) serum 1:10.
0.04 m*l* or diluted urine 1:100.
NH_3-free water to a volume of 0.97 m*l*.

Stir thoroughly and take two or three readings until the optical density remains constant. Add 0.01 m*l* glutamic dehydrogenase solution and stir. Read at five-minute intervals until no further decrease in optical density is observed, (20 min.). This decrease is due to the ammonia present. Add 0.02 m*l* of permutit-treated urease and stir. Read at five minute intervals until the reaction comes to an end, (20—30 min.). A "blank" cuvette containing all reagents except the "sample" is read simultaneously with the unknown and its value subtracted from that of the unknown. Optical measurments are taken at temperatures between 25—27° C.

Reagenziensätze für Analysen in einem Glas mit Asal-Monopacks



Serum pipettieren

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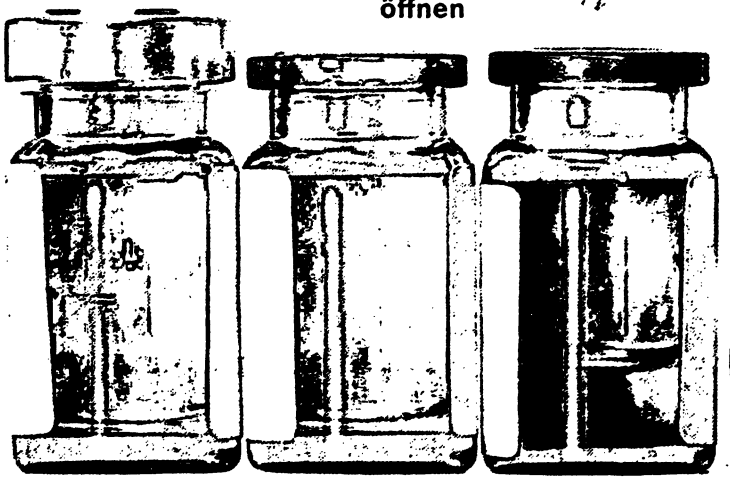

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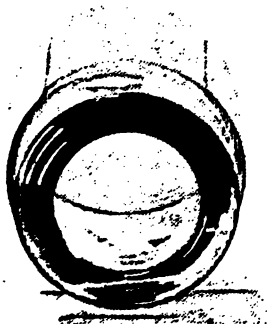

GOT-Monopack
Transaminaseaktivität
im Serum

GPT-Monopack
Transaminaseaktivität
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... Das Buch stellt ein Nachschlagewerk für jeden an der Immunitätslehre interessierten Forscher und Kliniker dar, für das nichts Vergleichbares im deutschen Schrifttum existiert. Seine besondere Eigenart liegt darin, daß dem Verfasser eine Synthese von experimenteller Forschung mit klinischer Immunologie gelang, für die der große Bereich der an klinischen Immunitätsfragen interessierten Ärzte besonders dankbar ist. Hennemann, Internist

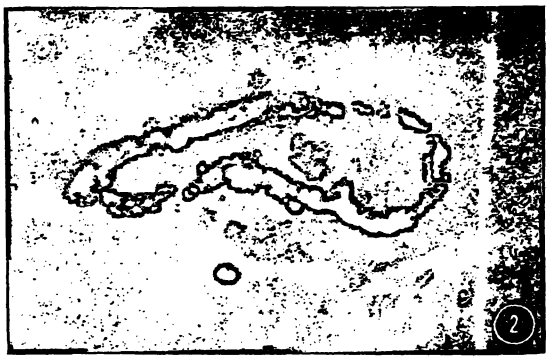
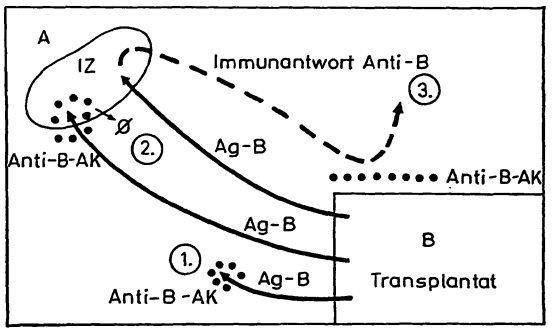


Abb. 35. Ablagerung präformierter, löslicher Antigen-Antikörper-Komplexe in der Wandung von Gefäßen bei anaphylaktischem Schock

Bronchusnahe gelegene Pulmonalgefäße von Meerschweinchen, bei denen zirkulierende Antigen-Antikörper-Komplexe (Rinder-Serumalbumin + Anti-Rinder-Serumalbumin vom Kaninchen) während eines anaphylaktischen Schocks zur Abbindung gelangten. 2: Behandlung mit fluoreszierendem Anti-Rinderserumalbumin (Nachweis des Antigens der Komplexe)

(Aus COCHRANE, Ch.: J. exp. Med. 118 [1963], 489)



V. Theorien über den Mechanismus des Enhancements

Abb. 58. Schematischer Vergleich von Transplantationsreaktion, Enhancement und Runt-Krankheit

A, B = Kennzeichnung des genetischen Unterschiedes zweier Inzuchtstämme als Empfänger und Spender von Transplantaten. IZ = immunologische Zentren (z. B. regionärer Lymphknoten); AG=Antigen, AK= Antikörper

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Calculation

Example: Sample = 0.05 m*l* of diluted serum (1:10).
Net decrease in optical density (ΔE) after correcting for the blank = 0.322.
The optical density at 340 nm of a solution containing one micromole of NADH per m*l* (light path 1 cm) is taken as 6.22. The urea *nitrogen* content per 100 m*l* serum is $\frac{0.322}{6.22} \times 14 \times 20 \times 10 \times 100 \times 0.001 = 14.49$ mg.

Results und Discussion

The validity of a quantitative method can best be tested by adding varying amounts of the substance to be measured into the material to be analysed. In this case, urea was added to normal serum to produce urea nitrogen concentrations of up to 250 mg/100 m*l* serum which is the highest one can anticipate in sera of uraemic patients. It can be seen from Table 1 that the recoveries were satisfactory and well within the technical errors expected with the use of a Beckman B spectrophotometer and the "ADH" graduated pipette used in clinical chemical laboratories. There is every reason to believe that the accuracy of the method can be further increased by using precision pipettes to measure the test sample and better spectrophotometers than the Beckman B instrument.

Table 1
Recovery of varying amounts of urea added to normal serum

	Amount of urea added (mg. Urea-N/100 m*l* serum)	% Recovery
Serum 1	93.8	102.8
	131.4	98.9
	168.9	99.7
	206.5	101.2
Serum 2	57.4	103.3
	95.6	102.6
	143.4	104.9
	191.2	102.7
	248.6	102.7

Furthermore, the enzymatic method was compared with the diacetyl monoxime method used in our Hospital Laboratory with the "Technicon" to determine serum urea (4). This colorimetric method utilizes dialysates of serum. The agreement between both methods was satisfactory in all ranges of urea concentrations. The use of the enzymatic method to determine urea (and ammonia) in urine presents no difficulties. However, if ammonia in urine is also to be measured, both substances cannot be determined in a "sequential" run on the same sample. Two different dilutions of the urine must be made, one for ammonia and one for urea, since the concentration of urea nitrogen is almost ten times that of ammonia nitrogen. Needless to say, when ammonia alone is to be determined in urine no addition of urease is necessary and the decrease in optical density after adding glutamic dehydrogenase is used for the calculation. Again, comparison of the enzymatic method for urinary urea with one of the colorimetric methods based on BERTHELOTS reaction of ammonia with hypochlorite and phenol (5) yielded satisfactory agreement between both methods.

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